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Apoptosis Induction by T-2 Toxin: Activation of Caspase-9, Caspase-3, and DFF-40/CAD through Cytosolic Release of Cytochrome *c* in HL-60 Cells

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The molecules participating in apoptosis induced by T-2 toxin in human leukemia HL-60 cells were investigated. The rank order of the potency of trichothecene mycotoxins to induce internucleosomal DNA fragmentation was found to be T-2, satratoxin G, roridin A >> diacetoxyscirpenol > baccharin B-5 >> nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X, baccharin B-4 = vehicle control. Western blot analysis of caspase-3 in T-2-treated cells clearly indicated the appearance of its catalytically active fragment of 17-kDa. Increased caspase-3 activity was also detected by using a fluorogenic substrate, DEVD-AMC. Next, cells exposed to T-2 led to cleavage of PARP from its native 116-kDa form to the 85-kDa product. Moreover, DFF-45/ICAD were cleaved to give a 12.5-kDa fragment *via* T-2 treatment. T-2 caused the release of cytochrome *c* from mitochondria into the cytosol. Increased enzymic activity of caspase-9 on LEHD-AMC was shown. These data indicate that T-2-induced apoptosis involves activation of caspase-3 and DFF-40/CAD through cytosolic accumulation of cytochrome *c* along with caspase-9 activation.

Key words: trichothecene mycotoxins; apoptosis; caspase-3; DFF-45/ICAD; cytochrome *c*

Strains of *Fusarium* species, which are common environmental contaminants of agricultural products such as maize, wheat, and oats, produce T-2 toxin (T-2).¹⁾ This sesquiterpenoid mycotoxin has the tetracyclic 12,13-epoxytrichothecene skeleton as shown in Fig. 1. A common toxicologic manifestation in humans as well as in various laboratory and food animal species following T-2 exposure is im-

munosuppression.^{2,3)} We reported that mice exposed to T-2 developed a prominent thymic atrophy through apoptotic cell death of thymocytes.^{4,5)} As regards the thymic apoptosis mechanism, we demonstrated that neither endogenous glucocorticoids nor tumor necrosis factor- α was involved.⁴⁾ We also showed that the Fas/Fas ligand system was not involved in the thymic apoptosis induced by T-2 *in vivo* in mice.⁶⁾

Apoptosis-inducing stimuli are of a great variety: X-ray irradiation, ultraviolet illumination, reactive oxygen species, Fas ligand, and chemicals such as anti-cancer drugs. The signaling pathway of apoptotic cell death is also diverse, in other words, the pathway used is greatly affected by the kind of stimuli. For the apoptotic process, 3 major pathways have been established: i) signal(s) caused by apoptotic stimulus acts on mitochondria to release cytochrome *c* to activate caspases,⁷⁾ ii) mitochondrion-bypass pathway that activates caspases,⁸⁾ iii) caspase-independent pathway.⁹⁾

Although we¹⁰⁾ and others¹¹⁾ reported that T-2 induces apoptosis in HL-60 cells, no information concerning the pathway of T-2-mediated apoptosis is available. We now demonstrate for the first time that activation of caspase-9, caspase-3, and DFF-40/CAD through cytosolic release of cytochrome *c* from the intermembrane space of mitochondria are involved in T-2-induced apoptosis in the experimental system described here.

Materials and Methods

Mycotoxins and reagents. T-2 and roridin A with

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Abbreviations: T-2, T-2 toxin; PARP, poly(ADP-ribose) polymerase; DFF-45/ICAD, DNA fragmentation factor-45/inhibitor of caspase-activated DNase; DFF-40/CAD, DNA fragmentation factor-40/caspase-activated DNase; HRPO, horseradish peroxidase; DMSO, dimethylsulfoxide; PBS(–), Dulbecco's phosphate buffered saline (without Mg²⁺ and Ca²⁺, pH 7.3); DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-methyl-coumarin; LEHD-AMC, acetyl-Leu-Glu-His-Asp-amino-methyl-coumarin; AMC, 7-amino-4-methyl-coumarin; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol-bis(β -aminoethyl ester) *N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase

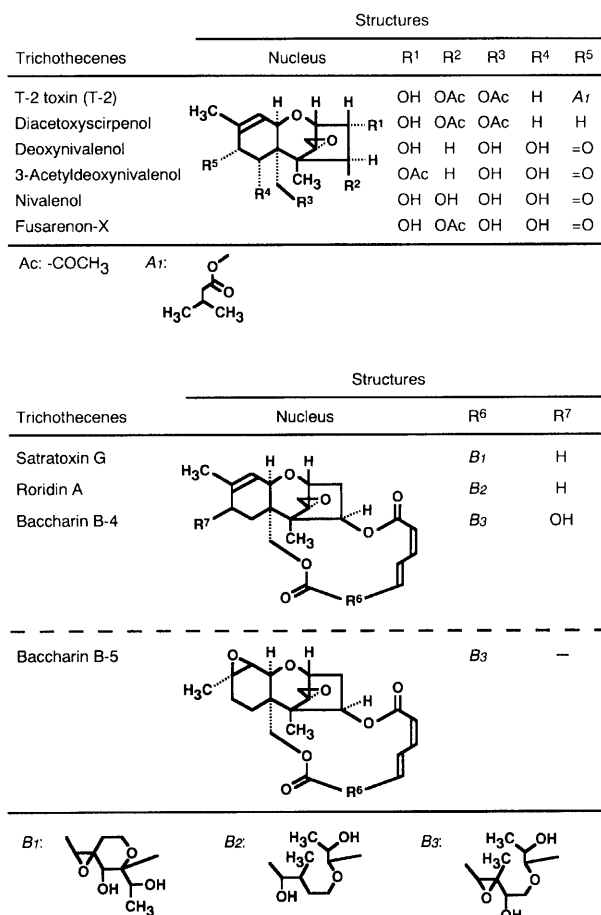


Fig. 1. Structures of Trichothecenes Used in This Study.

purity of over 98% were purchased from Sigma Chemical Company (St. Louis, MO, USA). Trichothecene mycotoxins including diacetoxyscirpenol, deoxynivalenol, 3-acetyldeoxynivalenol, nivalenol, fusarenon-X, and satratoxin G were prepared and purified by procedures reported previously.¹²⁻¹⁵ The purity of these mycotoxins was over 97% as measured by HPLC for satratoxin G and by GLC for the others. Baccharins B-4 and B-5 were kindly provided by B. B. Jarvis (University of Maryland, MD, USA). Mouse anti-CPP32/HRPO monoclonal antibodies, mouse anti-DFF45/ICAD monoclonal antibodies, mouse anti-PARP monoclonal antibodies, mouse anti-cytochrome *c* monoclonal antibodies (clone: 7H8.2C12), and rabbit anti-mouse IgG-HRPO were from Transduction Laboratories (Lexington, KY, USA), Medical & Biological Laboratories Co. (Nagoya, Japan), Affinity Bioreagents (Golden, CO, USA), BD Pharmingen (San Diego, CA, USA), and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Acetyl-Asp-Glu-Val-Asp-amino-methyl-coumarin (DEVD-AMC) and acetyl-Leu-Glu-His-Asp-amino-methyl-coumarin (LEHD-AMC) were from Peptide Institute (Minoh, Japan).

Cell culture. The HL-60 cell line used in this study was obtained from Riken Cell Bank (Tsukuba, Japan) and routinely cultured under a humidified atmosphere of 5% CO₂-95% air at 37°C in RPMI 1640 medium (Asahi Technoglass, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml).

Detection of DNA fragmentation by gel electrophoresis. DNA fragmentation was detected by the method as described.⁴

Assay for caspase activities. Activities for caspase-3 and caspase-9 were measured by the method described previously.¹⁶ In brief, cells (3×10^6) treated with T-2 were washed with PBS(-) and then resuspended in 750 µl of the lysis buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 10 mM EGTA; 20 µM digitonin). After incubation at 37°C for 10 min, the lysates were centrifuged at $16,000 \times g$ for 5 min. The supernatant was mixed with 250 µl of glycerol and then stored at -80°C for further use. A portion of each sample was incubated with 100 µM of DEVD-AMC or LEHD-AMC for caspase-3 or caspase-9, respectively, at 37°C for 1 h. The release of 7-amino-4-methyl-coumarin (AMC) was monitored using an MTP-100F fluoromicroplate reader (Corona Electric, Hitachi, Japan), using an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

Western blotting. The nuclear and cytosolic proteins for Western blotting of caspase-3,¹⁷ DFF-45/ICAD,¹⁸ and cytochrome *c*¹⁹ were prepared as described. Sample protein for PARP was prepared as described in the manufacturer's instructions. Protein concentration was measured by Protein Assay Dye (Bio-Rad Laboratories; Hercules, CA, USA). An equal volume of SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8; 2% 2-mercaptoethanol; 2% SDS; 40% glycerol; 0.1% bromophenol blue) was added and the proteins were boiled for 5 min. This preparation was deposited in the lanes of an SDS-polyacrylamide minigel and run at a constant current of 20 mA/gel. Following electrophoresis, proteins on the gel were electrotransferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia; Uppsala, Sweden) with a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories; Hercules, CA, USA) in 25 mM Tris, 198 mM glycine, 20% methanol. The membranes were then immunoblotted with primary antibodies (anti-human PARP antibody at a dilution of 1:2000, anti-DFF-45/ICAD antibody at a dilution of 1:1000, and anti-cytochrome *c* antibody at a dilution of 1:500) at room temperature for 1 h. After they were washed with PBS(-) containing 0.05% Tween 20 (PBS-T) for 5 min twice, the membranes were incubated with a rabbit anti-mouse IgG-

HRPO in PBS-T for 1 h at room temperature and then washed (twice, 5 min in PBS-T). In the case of caspase-3, the membranes were incubated with anti-human CPP32/HRPO at a dilution of 1:1000 for 1 h at room temperature, then rinsed (twice, 5 min in PBS-T). Detection was done by measuring the chemiluminescence of the ECL reagent (Amersham Pharmacia) with an LAS1000 system (Fuji Film, Tokyo, Japan).

Results

Effects of different kinds of trichothecene mycotoxins on internucleosomal DNA fragmentation in HL-60 cells

DNA fragmentation patterns induced by various trichothecene mycotoxins (structures: Fig. 1) at the same dose (20 nM) were studied by agarose gel electrophoresis (Fig. 2). A substantial amount of DNA was cleaved into a ladder of discrete fragments for T-2 (lane 2), satratoxin G (lane 8), and roridin A (lane 9). Diacetoxyscirpenol (lane 3) and baccharin B-5 (lane 11) induced apoptosis marginally, but essentially no DNA fragmentation was obtained for deoxynivalenol (lane 4), 3-acetyldeoxynivalenol (lane 5), nivalenol (lane 6), fusarenon-X (lane 7), or baccharin B-4 (lane 10). Based on our published results of dose and time course experiments,¹⁰ T-2 at a concentration of 200 nM and various time points as indicated in the figures were chosen for subsequent studies.

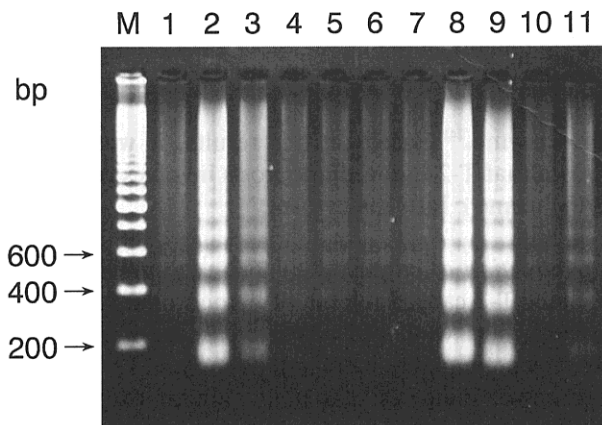


Fig. 2. DNA Fragmentation in HL-60 Cells Treated with Various Trichothecene Mycotoxins.

Cells were exposed to 20 nM of various trichothecene mycotoxins for 6 h: lane 1, vehicle control (0.1% DMSO); lane 2, T-2; lane 3, diacetoxyscirpenol; lane 4, deoxynivalenol; lane 5, 3-acetyldeoxynivalenol; lane 6, nivalenol; lane 7, fusarenon-X; lane 8, satratoxin G; lane 9, roridin A; lane 10, baccharin B-4; lane 11, baccharin B-5. DNA was electrophoresed (9.1×10^4 cells per lane) by the procedure described under Materials and Methods. Molecular size markers are shown on the left (M).

T-2 exposure activates caspase-3

As Z-VAD-FMK, a broad-range caspase inhibitor, inhibited internucleosomal DNA fragmentation by T-2 exposure (data not shown), activation of caspase-3 was investigated to test the involvement of caspase-3 in T-2-induced apoptosis (Fig. 3). During apoptosis, an inactive zymogen (CPP32) having 32-kDa is cleaved into a catalytically active 17-kDa fragment, caspase-3.²⁰ In cells treated with T-2, a band corresponding to 17-kDa was detected as early as 1 h (Fig. 3A). Furthermore, caspase-3 activity increased as early as 1 h (Fig. 3B). It is well known that PARP, one of the substrates of caspase-3, is characteristically processed during apoptosis from its native 116-kDa form to a truncated 85-kDa product.²¹ Treatment with T-2 cleaved PARP (Fig. 4). Taken together, it was clearly shown that T-2-induced apop-

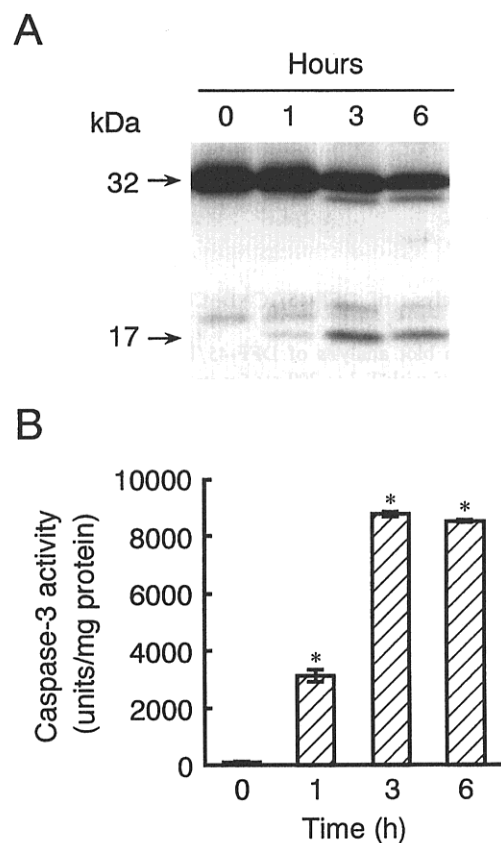


Fig. 3. Activation of Caspase-3 in T-2-Treated Cells.

A: Western blot analysis of caspase-3 activation in HL-60 cells treated with T-2 at 200 nM for indicated time points. Protein preparation, SDS-PAGE, electrotransfer and antibody staining are described under Materials and Methods. Thirty micrograms protein per lane were put on. Data are representative of two separate experiments. **B:** Caspase-3 activity was measured with DEVD-AMC. HL-60 cells were treated with 200 nM T-2 for indicated time points. Sample preparation and measurement of caspase activities are described under Materials and Methods. One unit was defined as the amount of enzyme required to release 0.44 pmol AMC per min at 37°C. Data were expressed as means \pm SE of three different determinations. *Significant difference from the data at 0 h: $p < 0.01$.

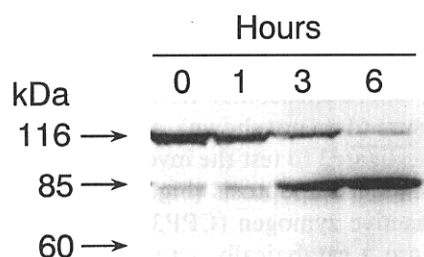


Fig. 4. Cleavage of PARP during T-2-Induced Apoptosis.

Western blot analysis of PARP cleavage in HL-60 cells treated with 200 nM T-2 for indicated time points. Protein corresponding to 2×10^5 cells were put on to each lane. Data are representative of two separate experiments.

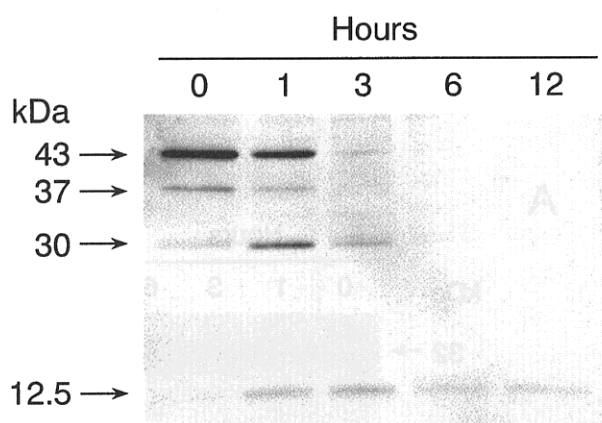


Fig. 5. Analysis of DFF-45/ICAD Cleavage in Cells Treated with T-2.

Western blot analysis of DFF-45/ICAD cleavage in HL-60 cells treated with T-2 at 200 nM for indicated time points. Seven and a half micrograms of protein were put on to each lane. Data are representative of two separate experiments.

tosis involves activation of caspase-3.

T-2-stimulus induces cleavage of DFF-45/ICAD

It has been reported that caspase-3 activated by apoptosis signals cleaves DFF-45/ICAD to release DFF-40/CAD, which has DNA fragmentation activity.^{22,23} To test whether such cleavage is actually occurring in T-2-induced apoptosis, Western blotting was done. As shown in Fig. 5, a degraded fragment (12.5-kDa) of DFF-45/ICAD (43- and 37-kDa)²³ could be detected. The 30-kDa band, an intermediate degradation product, disappeared after 6 h (Fig. 5). These data indicate that DFF-45/ICAD degradation, in other words, activation of DFF40/CAD, is involved in T-2-mediated apoptosis.

T-2 causes cytosolic cytochrome c release

To find whether a mitochondria-dependent pathway is operating or not in this system, cytosolic cytochrome *c* was analyzed. As shown in Fig. 6, significant accumulation of cytosolic cytochrome *c* was observed. A faint band at 0 h should be apo-cytochrome *c* without heme. Cytochrome *c*-induced apoptosis was dependent on heme attachment to the

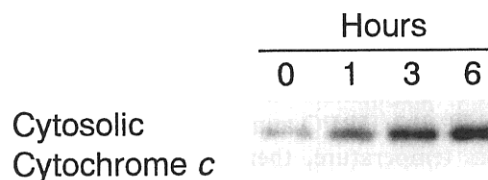


Fig. 6. Cytosolic Accumulation of Cytochrome *c*.

Western blot analysis of cytochrome *c* releasing into the cytosol in HL-60 cells treated with 200 nM T-2 for indicated time points. One microgram of protein were put on to each lane. Data are representative of two separate experiments.

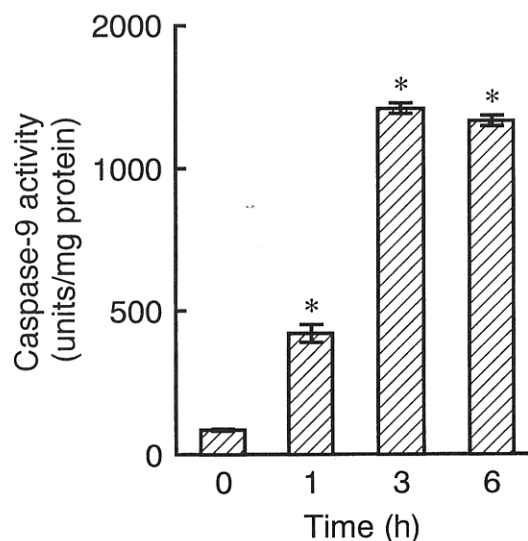


Fig. 7. Caspase-9 Activity on LEHD-AMC in T-2-Treated Cells.

Caspase-9 activity was measured with LEHD-AMC. Cells were treated with 200 nM T-2 for indicated time points. One unit was defined as shown in Fig. 3. Data were expressed as means \pm SE of three different determinations. *Significant difference from the data at 0 h: $p < 0.01$.

apo-enzyme.²⁴ Based on our results, it was clearly shown that T-2-induced apoptosis involves release of cytochrome *c* into the cytosol.

Activation of caspase-9 by T-2-exposure

Since T-2 caused release of cytochrome *c* into the cytosol, next we tested whether caspase-9 is activated. The activity was increased as early as 1 h (Fig. 7). The result indicates that activation of caspase-9 is involved in T-2-mediated apoptosis.

Discussion

Apoptosis, or cellular suicide, is necessary to purge cells that are in excess, in the way, or potentially dangerous. To this end, multicellular animals use specialized cellular machinery. Recently, a variety of molecules participating in the biochemical pathway that mediates the process of apoptosis have been identified.²⁵ The signaling pathway of apoptotic cell death is not single, but diverse: there have been at least 3

known major pathways²⁵⁾ including the mitochondria-bypass pathway⁸⁾ as mentioned in the introduction of this report. Recently, 2 groups have described the molecular mechanisms of apoptosis triggered by trichothecenes using different cell lines.^{26,27)} However, it remained unresolved which pathway among the 3 pathways is used in their experimental system. Here we found that apoptosis induced by a trichothecene mycotoxin, T-2 toxin, involves activation of caspases through release of cytochrome *c* from mitochondria into the cytosol. Various apoptosis-inducing stimuli, but not all, induce cytosolic cytochrome *c* from mitochondria, where it binds to the Apaf-1, inducing binding to procaspase-9 and resulting in activation of caspase-9. Active caspase-9 then directly activates caspase-3, resulting in the proteolytic degradation of PARP and DFF-45/ICAD.⁷⁾ Our present finding of this T-2-induced apoptotic pathways supports the events of apoptotic pathway published by other groups.^{28,29)} Although internucleosomal DNA fragmentation is executed by at least DFF-40/CAD in this experimental system, further studies are needed to clarify the possible involvement of other endonucleases³⁰⁾ than DFF-40/CAD.

The signaling mechanism of apoptosis triggered by T-2 exposure is still obscure. Although the role of c-Jun N-terminal kinase (JNK) in apoptosis is controversial,^{31–33)} the JNK signaling pathway has been implicated in the apoptotic response of cells exposed to stress.³⁴⁾ Recently, it has been reported that JNK and p38 mitogen-activated protein kinase (MAPK) may play important roles in apoptotic cell death caused by trichothecene mycotoxins in the different experimental system from ours.^{26,27)} However, the correlation between our present data and information concerning involvement of kinases in apoptosis is not understood. Further studies at the molecular level are necessary to clarify this point.

Taken together, our results and those of others^{26,27)} have established partial signaling events associated with cell death triggered by trichothecenes. Nevertheless, the biochemical reaction at the first stage of the apoptotic process remains to be elucidated. Many investigators have tacitly assumed that the 60S ribosomal subunits in eukaryotic cells would be the initial molecules for trichothecenes to inhibit protein synthesis.^{35,36)} However, there was no obvious relationship between the magnitude of apoptosis and potency to inhibit protein synthesis by various trichothecenes.²⁶⁾ Thus, at present, the molecular mechanism at the initial step of apoptosis induced by trichothecenes seems complex. But, notably, it is well known that trichothecenes are able to increase lipid peroxidation.^{37,38)} Recently, interesting papers supporting a correlation between lipid peroxidation and apoptosis have appeared.^{39,40)} Namely, it is reported that activation of caspase-3 followed by degradation of PARP is associated with apoptosis induced by free

radical-induced lipid peroxidation of cellular membrane.⁴⁰⁾ To answer the question whether lipid peroxidation plays an important role in the T-2-induced apoptosis in our experimental system awaits further investigations.

Although it has been reported that environmental toxic chemicals such as dioxin influence many critical life processes *via* receptors,⁴¹⁾ no information on T-2 toxin receptors is available. An attempt at molecular cloning to obtain cDNA encoding a T-2 receptor is in progress in our laboratory. Identification of T-2 receptors could contribute profoundly to delineate the apoptotic mechanism.

In conclusion, our study illustrates that caspase-9 activated *via* cytosolic release of cytochrome *c* activates caspase-3, resulting in fragmentation of nucleosomal DNA by DFF-45/CAD in T-2-induced apoptosis in HL-60 cells. Future work will be directed towards identifying upstream signals preceding mitochondria in trichothecene mycotoxin-induced apoptosis.

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